Liver fibrosis in mice induced by carbon tetrachloride and its reversion by luteolin

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Hepatic fibrosis is an effusive wound healing process in which excessive connective tissue builds up in the liver. Because specific treatments to stop progressive fibrosis of the liver are not available, we have investigated the effects of luteolin on carbon tetrachloride (CCl4)-induced hepatic fibrosis. Male Balb/C mice were treated with CCl4 (0.4 ml/kg) intraperitoneally (i.p.), twice a week for 6 weeks. Luteolin was administered i.p. once daily for next 2 weeks, in doses of 10, 25, and 50 mg/kg of body weight. The CCl4 control group has been observed for spontaneous reversion of fibrosis. CCl4-intoxication increased serum aminotransferase and alkaline phosphatase levels and disturbed hepatic antioxidative status. Most of these parameters were spontaneously normalized in the CCl4 control group, although the progression of liver fibrosis was observed histologically. Luteolin treatment has increased hepatic matrix metalloproteinase-9 levels and metallothionein (MT) I/II expression, eliminated fibrinous deposits and restored architecture of the liver in a dose-dependent manner. Concomitantly, the expression of gland fibroblastic acidic protein and α-smooth muscle actin indicated deactivation of hepatic stellate cells. Our results suggest the therapeutic effects of luteolin on CCl4-induced liver fibrosis by promoting extracellular matrix degradation in the fibrotic liver tissue and the strong enhancement of hepatic regenerative capability, with MTs as a critical mediator of liver regeneration.

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Introduction

Liver fibrosis is a frequent event which follows a repeated or chronic insult of sufficient intensity to trigger a “wound healing”-like reaction, characterized by excessive connective tissue deposition in extracellular matrix (ECM). Chronic carbon tetrachloride (CCl4) intoxication is a well-known model for producing oxidative stress and chemical hepatic injury. Its biotransformation produces hepatotoxic metabolites, the highly reactive trichloromethyl free radical, which are further converted to the peroxytrichloromethyl radical (Williams and Burk, 1990). Reactive oxidant species likely contribute to both onset and progression of fibrosis (Poli, 2000). Antioxidant treatment in vivo seems to be effective in preventing or reducing chronic liver damage and fibrosis (Parola and Robino, 2001). Polyphenols, naturally occurring antioxidants in fruits, vegetables, and plant-derived beverages such as tea and wine, have been associated with a variety of beneficial properties (Havsteen, 2002). The flavone luteolin (3′,4′,5,7-tetrahydroxyflavone) is an important member of the flavonoid family, present in glycosylated forms and as aglycone in various plants (Shimoi et al., 1998). Luteolin is reported to have anti-inflammatory (Ziyun et al., 2007; Veda et al., 2002), antioxidant (Perez-Garcia et al., 2000), antiallergic (Veda et al., 2002), antitumorigenic (Ju et al., 2007), anxiolytic-like (Coleta et al., 2008), and vasorelaxative properties (Woodman and Chan, 2004). Previously, we have shown a hepatoprotective activity of luteolin in acute liver damage in mice (Domitrovich et al., 2008a, Domitrovich et al., 2009).

Hepatic stellate cells (HSCs) are a minor cell type most commonly found in the space of Disse, intercalated between hepatocytes and cells lining the sinusoid, projecting their dendritic processes to nearby hepatocytes and endothelial cells (Blouin et al., 1977, Mermelstein et al., 2001). Upon liver injury, HSCs become activated, converting into myofibroblast-like cells. Activated HSCs proliferate and produce extracellular matrix (ECM), playing a major role in hepatic fibrosis and regeneration (Friedman, 2000). ECM, which consists of collagens and other matrix components such as proteoglycans, fibronectins, and hyaluronic acid (Arthur, 1994), is regulated by a balance of synthesis and enzymatic degradation of ECM. The key enzymes responsible for degradation of all the protein components of ECM and basement membrane are matrix metalloproteinases (MMPs), a zinc-dependent family of endopeptidases. Previous studies have demonstrated that the activity of these enzymes is altered during the processes of fibrogenesis and fibrinolysis (Knittel et al., 2000).

The metallothioneins (MTs), small cysteine-rich heavy metal-binding proteins, participate in an array of protective stress responses. In mice, among the four known MT genes, the MT I and MT II genes are...
most widely expressed. Transcription of these genes is rapidly and dramatically up-regulated in response to agents which cause oxidative stress and/or inflammation (Andrews, 2000). The induction of MT synthesis can protect animals from hepatotoxicity induced by various toxins including CCl₄, but also play a role in repair and regeneration of injured liver (Cherian and Kang, 2006).

Because the specific treatments to stop progressive fibrosis of the liver are not available, the objective of the present study was to investigate the therapeutic effect and mechanisms of action of luteolin in chemically induced liver fibrosis in mice.

Materials and methods

Materials. Luteolin, carbon tetrachloride (CCl₄), olive oil, dimethyl sulfoxide (DMSO), nitric acid (HNO₃), hydrogen peroxide (H₂O₂), and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of the highest grade commercially available.

Animals. Male Balb/c mice from our breeding colony, 2–3 months old, were divided into 6 groups with 5 animals per group. Mice were fed a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% ash, and 2.6% by weight of lipids (native soya oil) to prevent essential fatty acid deficiency. Total energy of the diet was 16.4 MJ/kg. The animals were maintained at 12 h light/dark cycle, at constant temperature (20±1 °C) and humidity (50±5%). All experimental procedures were approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

Experimental design. Mice were given CCl₄ intraperitoneally (i.p.) at a dose of 0.4 ml/kg, dissolved in olive oil, twice a week for 6 weeks (the CCl₄ group), except the control group which received vehicle only. Seventy-two hours after the last CCl₄ injection, the CCl₄ group was killed. The CCl₄ control group was observed for spontaneous resolution of hepatic fibrosis for next 2 weeks. In the luteolin-treated groups, the polyphenolic compound was administered i.p. at a dose of 10, 25, or 50 mg/kg daily for 2 weeks, respectively. These doses were selected on the basis of preliminary studies (Domitrović et al., 2008a, Domitrović et al., 2009). Luteolin was dissolved in DMSO and diluted in saline to the manufacturer’s instructions.

Hepatotoxicity study. Serum levels of ALT, AST, and ALP as markers of hepatic function, were measured by using a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) according to the manufacturer’s instructions.

Determination of Cu/Zn SOD activity and GSH concentration. Cu/Zn SOD activity and total GSH, indicators of oxidative stress, were measured spectrophotometrically, using Superoxide Dismutase Assay Kit and Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer’s instructions. Protein content in supernatants was estimated by Bradford’s method, with bovine serum albumin used as a standard (Bradford, 1976).

Histopathology. Liver specimens were fixed in 4% phosphate-buffered formalin, embedded in paraffin, and cut into 4 μm thick sections. Sections for histopathological examination were stained with hematoxylin and eosin (H&E) and Mallory trichrome stain using standard procedure.

Immunohistochemical determination of GFAP, α-SMA, and MT I/II. Immunohistochemical studies were performed on paraffin embedded liver tissues using mouse monoclonal anti-MT I+II antibody diluted 1:50 (clone E9; DakoCytomation, Carpinteria, CA, USA), mouse monoclonal anti-GFAP antibody diluted 1:100 (clone 1B4; BD Pharmingen, San Diego, CA, USA), and mouse monoclonal antibody to α-SMA diluted 1:100 (SPM332; Abcam, Cambridge, UK), employing DAKO EnVision+ System, Peroxidase/DAB kit according to the manufacturer’s instructions (DAKO Corporation, Carpinteria, CA, USA). Briefly, slides were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, monoclonal antibodies diluted in phosphate-buffered saline supplemented with bovine serum albumin were added to tissue samples and incubated overnight at 4 °C in a humid environment, followed by incubation with peroxidase labeled polymer conjugated to secondary antibodies containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction product was visualized by adding substrate–chromogen diaminobenzidine (DAB) solution, resulting with brownish coloration at antigen sites. Tissues were counterstained with hematoxylin, dehydrated in gradient of alcohol, and mounted with mounting medium. The intensity of staining was graded as weak, moderate, and intense. The specificity of the reaction was confirmed by substitution of primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX51, Tokyo, Japan).

MMP zymography. MMP-2 and MMP-9 activities were analyzed by gelatin zymography assays as described (Kuo et al., 2003), with modifications. After tissue homogenization in radioimmunoprecipitation assay buffer (4 ml of buffer per gram of tissue) containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 1 mM sodium orthovanadate, and 2 μg/ml of each aprotinin, leupeptin and pepstatin. 10 μg of liver tissue protein lysates were separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin, at 4 °C and 150 V for 4 h. Gels were washed for 30 min in 2.5% Triton X-100 to remove the SDS, and briefly being filtered through a 0.45-μm filter, 2 ml of samples was extracted and analyzed according to the procedure of Bergman and Lasley (1963). Briefly, sample neutralization was obtained with 10 M NaOH and 3 M HCl. After neutralization, subsequent steps were made in duplicate for each sample. To a 200 μl of the above solution, 400 μl of isopropanol in citrate-acetate-buffered Chloramine T was added. After 4 min, 2.5 ml of Ehrlich reagent was added. Tubes were wrapped in aluminum foil and incubated for 25 min in a water-bath at 60 °C, cooling each sample in tap water, and measuring the absorbance of each sample spectrophotometrically at 550 nm (Cary 100, Varian, Mulgrave, Australia).

Determination of trace elements. Hepatic zinc (Zn) and copper (Cu) content were determined by ion coupled plasma spectrometry (ICPS) using Prodigy ICP Spectrometer (Leeman Labs, Hudson, NH, USA), according to the method previously described (Domitrović et al., 2008a).

Determinantion of retinol. The hepatic levels of retinol were analyzed by high-performance liquid chromatography (HPLC) according to Hosotani and Kitagawa (2003), as described previously (Domitrović et al., 2008b).

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washed in the reaction buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.02% NaN₃. The reaction buffer was changed to a fresh one, and the gels were incubated at 37 °C for 48 h. Gelatinolytic activity was visualized by staining the gels with 0.1% Coomassie blue R-350, destained with methanol-acetic acid water (30:10:60 v/v) two times for 20 min. The clear zones in the background of blue staining indicate the presence of gelatinase activities. The intensity of the bands was assayed by scanning video densitometry (Bio-Rad GS-710 Calibrated Imaging Densitometer, Bio-Rad Laboratories, UK).

Statistical analysis. Data were analyzed using StatSoft STATISTICA version 7.1 software. Differences between the groups were assessed by a nonparametric Man–Whitney and Kruskal–Wallis tests. Values in the text are means±standard deviation (SD). Differences with p<0.05 were considered to be statistically significant.

Results

Hepatotoxicity

Serum AST, ALT, and ALP activities were changed significantly in mice receiving CCl₄ twice a week for 6 weeks and terminated 72 h later (Table 1). The relative liver weight significantly increased in the CCl₄ group when compared to controls. In the CCl₄ control group, observed for the spontaneous regression of fibrosis for 2 weeks, the relative liver weight and ALP activity were still increased; however, AST and ALT activities were normalized. Luteolin administration attenuated the elevation of ALP activity in the mice treated with CCl₄ and decreased relative liver weight to control values. Higher doses of luteolin, 25 and 50 mg/kg, decreased ALP activity below control values. However, AST activity in mice treated with 50 mg/kg of luteolin was significantly increased compared to the control group and groups treated with luteolin.

Hepatic hydroxyproline

The liver hydroxyproline content was fivefold higher in the CCl₄ group than in controls and progressively increased in the CCl₄ control group (Table 1). Luteolin therapy significantly decreased the hepatic hydroxyproline level, which was returned to normal values in the group receiving 50 mg/kg of luteolin.

Cu/Zn SOD activity and GSH concentration

There was no evidence of oxidative stress in the CCl₄ control group when compared to the CCl₄ group. The hepatic total GSH level remained at normal values throughout the treatment period, except in the group treated with 50 mg/kg of luteolin, where it was significantly increased (Table 2). Cu/Zn SOD activity was increased in the CCl₄ group; however, its level was normalized in the CCl₄ control group and groups treated with luteolin. The liver total retinol concentration was markedly decreased in the CCl₄ group, then increased in the CCl₄ control group, returning to normal values in the group treated with the highest dose of luteolin.

Cu and Zn content

The Cu content has not been significantly changed by CCl₄ intoxication or during luteolin therapy. The liver trace element content was expressed as μg/g dry liver weight (ppm).

Histopathology

Liver sections from control mice stained with hematoxylin and eosin (H&E) showed normal hepatic architecture. Liver specimens from the CCl₄ group showed remnants of degenerated and balloonned/necrotic hepatocytes containing acidophilic hyaline inclusions (Fig. 1B). Mild mononuclear cell infiltration in these areas was also present. Hepatocytes in the vicinity of hepatic lesions, particularly those situated immediately alongside the lesion border, showed features of pronounced macrovesicular or microvesicular steatosis. Distended sinusoidal spaces filled with erythrocytes as a morphological hallmark of congestion (disturbed blood flow) were also observed. Hepatic lesions, fatty degeneration, and hyaline deposits as well as morphological signatures of congestion were more pronounced in the CCl₄ control group (Fig. 1C). Hepatic lesions and hyaline deposits were also present in the livers of mice treated with 10 mg/kg of luteolin (Fig. 1D), but they were significantly reduced in extent and were less frequent compared to the CCl₄ control group, whereas macrovesicular steatosis was still present in the surrounding liver parenchyma, with noticeable signs of congestion. The livers of mice receiving 25 mg/kg of luteolin showed only sporadic, markedly small hepatic lesions, hyaline

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Each value represents the mean±SD for 5 mice. Values not sharing a common letter superscript (a, b, c) are significantly different (p<0.05). Statistical difference was determined using nonparametric Man–Whitney and Kruskal–Wallis tests.
changes, and microvesicular steatosis in the periportal areas, but without morphological signs of hemodynamic disturbance (Fig. 1E). In animals treated with 50 mg/kg of luteolin, the livers showed maintained histoarchitecture, almost similar to controls, with only weak microvesicular steatosis of periportal and perilobular hepatocytes (Fig. 1F). There were no changes which could be attributed to hepatic hemodynamic disturbances in these animals.

The livers from control mice stained with Mallory trichrome stain showed traces of collagen only in the vascular walls. Liver section from the CCl4 group showed multiple fibrotic nodules and extensive fibrosis predominantly in the periportal areas (Fig. 2B). The pericellular collagen deposition and the extent of fibrotic changes were even more pronounced in the CCl4 control group (Fig. 2C). Fibrotic lesions were still present in the livers of mice treated with 10 mg/kg of luteolin (Fig. 2D), but they were significantly reduced in extent and were less frequent compared to the CCl4 control group, whereas mild pericellular fibrosis was observed in the surrounding liver parenchyma. The livers of mice receiving 25 mg/kg of luteolin showed only sporadic, markedly

Fig. 1. The effect of CCl4 and luteolin therapy on liver histology. (A) The livers from control mice showed normal hepatic architecture. (B) The livers of mice receiving CCl4 twice a week for 6 weeks, terminated 72 h after the last CCl4 injection, showed severe hepatic lesions, degenerated and ballooned necrotic hepatocytes with cellular hyaline inclusions, and mild mononuclear cell infiltration (arrowheads). (C) In the CCl4 control group, observed for spontaneous regression of fibrosis for additional 2 weeks, hepatic lesions, ballooned hepatocytes, and hyaline bodies were more frequent. (D) Hepatic lesions and hyaline deposits in mice receiving 10 mg/kg luteolin for 2 weeks were significantly reduced in extent and were less frequent. (E) The livers of mice receiving 25 mg/kg of luteolin showed sporadic, small hepatic lesions, hyaline changes, and microvesicular steatosis. Arrows show ballooned hepatocytes with hyaline inclusions. Original magnification 100×, insets 1000×. H&E stain.
small fibrotic deposits in the periportal areas, with a discrete pericellular fibrosis (Fig. 2E). Animals treated with 50 mg/kg of luteolin showed only minor and sporadic pericellular fibrotic changes (Fig. 2F).

**Immunohistochemistry of GFAP, α-SMA, and MT I/II**

Activated HSCs could be distinguished from the other myofibroblasts of the liver (such as portal myofibroblasts) by their specific position in the liver parenchyma (Cassiman et al., 2002). With regard to the distribution of α-SMA-positive fibrogenic cells, in the livers of control animals, α-SMA immunopositivity was restricted to the smooth musculature belonging to the arterial tunica media, as well as to the wall of majority of portal and central veins, while other liver cells remain negative (Fig. 3A). CCl₄ strongly induced perisinusoidal α-SMA expression, which was recognized as activated HSCs, through affected lobuli, connected between themselves with thin, “bridging” immunopositivity (Fig. 3B). The HSCs varied in shape and size. In the CCl₄ control group, perisinusoidal immunopositivity was withdrawn, but emerged in hepatocytes located at the scar-parenchyma interface (Fig. 3C). The livers of mice receiving 10 and 25 mg/kg of luteolin showed only

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**Fig. 2.** The histopathologic detection of collagen in the livers. Collagen fibers of the connective tissues are identified by their blue color. (A) Liver section from a control mice. (B) Mice receiving CCl₄ twice a week for 6 weeks, terminated 72 h after the last CCl₄ injection, had developed extensive fibrosis in the periportal areas. (C) The CCl₄ control group, observed for spontaneous regression of fibrosis for additional 2 weeks, had more intense collagen staining. (D) The collagen deposits in mice receiving 10 mg/kg luteolin for 2 weeks were greatly reduced in size. (E) The livers of mice receiving 25 mg/kg of luteolin showed sporadic, small fibrotic lesions in the periportal zone. (F) In mice treated with 50 mg/kg of luteolin there were observed only minor pericellular fibrotic changes (arrow). Original magnification 100×, insets 1000×. Mallory trichrome stain.
sporadic α-SMA immunopositivity in the hepatocytes in the vicinity of fibrotic lesions (Figs. 3D and E). The livers of mice receiving 50 mg/kg of luteolin showed staining pattern similar to control animals.

Immunohistochemical staining using monoclonal anti GFAP antibody showed thin, irregular immunopositive bands lining the hepatic sinusoids of control animals (staining pattern considered as a characteristic for HSC) (Fig. 4A). In the CCl4 and CCl4 control groups, moderate and strong GFAP expressions, respectively, were limited to the scar areas (Figs. 4B and C). Treatment with 10 and 25 mg/kg of luteolin decreased GFAP expression in the fibrotic areas, but induced sporadic assembling of GFAP positive cells in the surrounding tissue (Figs. 4D and E). The livers of mice receiving 50 mg/kg of luteolin showed staining pattern similar to control animals, characterized by moderate GFAP expression alongside the sinusoidal border (Fig. 4F).

Control animals had moderate cytoplasmic MT I/II expression in the perilobular areas (Fig. 5A). Liver tissues from the CCl4 group showed weak cytoplasmic immunostaining of fatty changed hepatocytes surrounding the fibrotic areas, but strong immunopositivity within balloonized hepatocytes (Fig. 5B). In the CCl4 control group, MT I/II immunopositivity was found to be only sporadic and restricted to

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**Fig. 3.** The expression and specific tissue distribution of α-SMA. (A) In the livers of control animals, α-SMA immunopositivity was restricted to smooth musculature (arrow), while other liver cells remain negative. (B) In mice receiving CCl4 twice a week for 6 weeks, terminated 72 h after the last CCl4 injection, many α-SMA-containing myofibroblasts were strongly and diffusely stained in affected lobuli, connected between themselves with thin, "bridging" immunopositivity (arrows). (C) In the CCl4 control group, observed for spontaneous regression of fibrosis for additional 2 weeks, clusters of moderately α-SMA-immunopositive hepatocytes located at the scar-parenchyma interface were found (arrows). The livers of mice receiving 10 mg/kg (D) and 25 mg/kg (E) of luteolin showed only sporadic α-SMA immunopositivity in the hepatocytes in vicinity to fibrotic lesions (arrows). (F) The livers of mice receiving 50 mg/kg of luteolin showed staining pattern similar to control animals. Original magnification 400×, inset 1000×. Immunohistochemical stain for α-SMA.
hyaline bodies in the context of fibrous lesions, while in the remnant liver tissue was not detected (Fig. 5C). The livers of animals receiving 10 mg/kg of luteolin showed weak cytoplasmic MT I/II expression in the remaining hepatic parenchyma and more frequent appearance of MT I/II immunopositive hyaline deposits (Fig. 5D). Concomitantly with the histoarchitecture improvement, moderate perilobular MT I/II immunopositivity was observed in the livers of animals treated with 25 mg/kg of luteolin (Fig. 5E). Liver sections from mice treated with 50 mg/kg of luteolin have shown strong cytoplasmic and nuclear immunopositivity, remarkably in the periportal and perilobular areas (Fig. 5F). Fig. 5G shows the extent of MT I/II expression.

MMP zymography

MMPs were identified by their molecular weights. Liver extracts contained mainly the latent form of MMP-9 at about 92 kDa (Fig. 6). CCl₄-intoxication decreased MMP-9 expression, which was even more pronounced in the CCl₄ control group. Treatment with luteolin resulted in an increase in MMP-9 expression in a dose-dependent manner, being the most prominent in mice treated with 25 and 50 mg/kg of luteolin. Active form of MMP-9 was barely detectable in groups treated with 25 and 50 mg/kg of luteolin. In contrast to MMP-9, both latent and active forms of MMP-2 expression were below detection limit in all groups.
or degraded by proteasomes (Kopito, 2000). However, if reparation or degradation fails, abnormal proteins became segregated in the cytoplasm as inclusion bodies.

Hepatic indicators of oxidative stress and hepatic damage in this study were markedly changed in CCl4-intoxicated group, 72 h after discontinuation of the toxin. Two weeks later, in the CCl4 control group and the groups treated with luteolin, most of the biochemical parameters, Cu/Zn SOD activity, GSH, and aminotransferase levels did not indicate significant oxidative stress or hepatocellular damage. The possible reason for this is that mammalian cells respond quickly to the initial oxidative damage by means of a characteristic adaptive phenomenon (Radice et al., 1998). Acquisition of adaptation to tissue damage is a common response to insults in order to reduce the vulnerability of the cell or tissue, which depends on the activity of various stress sensors, signal transduction and effectors of protection (Hagberg et al., 2004). Therefore, biochemical parameters may not reflect the degree of tissue damage (Murayama et al., 2007). These results are of the significant clinical importance, suggesting that the classical biochemical parameters such as serum aminotransferase activities are not a reliable indicators of chronic liver damage.

The accumulation of ECM observed in fibrosis and cirrhosis is considered to be the result of activation of fibroblasts, which acquire a myofibroblastic phenotype. Myofibroblasts are produced by the activation of precursor cells, such as HSCs and portal fibroblasts. Significant differences between these two fibrogenic cell populations have been reported, in the mechanisms leading to myofibroblast differentiation, their activation and deactivation (Guyot et al., 2006). However, the pathophysiological mechanisms of hepatic fibrogenesis are not yet fully understood. In particular, the role of HSCs remains unclear. HSCs have been also found to migrate in vitro (Marra et al., 1999), suggesting that they may migrate to the fibrotic lesions and take part in the repair process (Kinnamon et al., 2000). Stimulation of HSCs with various cytokines or type I collagen, results in an increase in their migratory capacity, which could be accompanied by or independent of HCS proliferation (Yang et al., 2003). In the quiescent state, HSCs express desmin and GFAP (Yokoi et al., 1984, Neubauer et al., 1996). Upon HSCs activation, GFAP expression decreases, whereas the levels of desmin and α-SMA increase, due to their transformation into myofibroblast-like cells (Campbell et al., 2005). In this study, deposition of collagen in damaged hepatic areas, associated with increased α-SMA and decreased GFAP perisinusoidal immunopositivity, indicates that activated HSCs are responsible for the fibrosis seen in the CCl4-intoxicated mice. Additionally, increased GFAP immunopositivity in fibrotic scars suggests the accumulation of quiescent HSCs at the places of active fibrogenesis. Changes in the activation marker expression in the CCl4 control group, however, indicate deactivation of HSCs, which is critical for resolution of fibrosis (Iredale, 2001). Nevertheless, α-SMA immunopositive hepatocytes located at the scar-parenchyma interface in the CCl4 control group suggest that hepatocytes have acquired myofibroblast-like phenotype. Indeed, Zeisberg et al. (2007) have recently shown that adult hepatocytes could be actively engaged in the epithelial to mesenchymal transition mediated by TGF-β1, by expressing fibroblast-specific protein-1 (FSP1), suggesting that hepatocyte-derived fibroblasts could be an additional lineage of mesenchymal cells that contribute to the progression of liver fibrosis. Furthermore, Dooley et al. (2008) have observed hepatocyte transdifferentiation immediately adjacent to the fibrotic lesions, which is in agreement to our findings. However, to our knowledge, this is for the first time that the α-SMA expression,
characteristic for activated HSCs, is found in hepatocytes. This subpopulation of hepatocytes could contribute to collagen deposition observed in the CCl₄ control group. Perisinusoidal GFAP expression in the group treated with 50 mg/kg of luteolin indicates a complete return of HSCs into a quiescent state.

Morphological studies have shown that fibrotic lesion of the liver also typically involves, beside an accumulation of extracellular collagen matrix and a transformation of stellate cells to actively dividing myofibroblasts, a reduction in stellate cell lipid droplets and hepatic storage of total retinol (Blomhoff and Wake, 1991). Decreased hepatic total retinol level in the CCl₄ group observed in this study is in agreement with previous studies (Seifert et al., 1994, Natarajan et al., 2005). The vitamin A status of the liver plays an important role in hepatic fibrogenesis. The reduction of retinoid levels in HSC by an increased secretion of retinol from the liver into the circulation during CCl₄ treatment may stimulate the transformation of these cells into fibroblasts and contribute to fibrogenesis (Seifert et al., 1994). Increased liver retinol observed in the CCl₄ control group, as well as in mice receiving luteolin therapy, also indicates a decrease in the number of activated HSCs.

In normal tissues, matrix protein degradation is accomplished by a family of MMPs, whose expression significantly increases in the case of acute liver injury (Arthur, 1994). In chronic liver injury sustained tissue damage and inflammation also induce MMPs overexpression (Kossakowska et al., 1998). MMP-2, the 72-kD collagenase IV/gelatinase A, is an important factor in the metabolism of type IV collagen in sinusoid basement, produced by HSCs. MMP-9, the 92-kD collagenase IV/ gelatinase B, a major MMP in basement membrane-like ECM remodeling, is secreted by neutrophil granulocytes, macrophages and leukocytes as a major source for MMP-9 expression (Salguero Palacios et al., 2008), with HSCs as an additional source (Kniittel et al., 1999). MMP activity decreases with the progression of liver fibrosis (Iredale, 2001). Liver injury which results in HSC activation, particularly in chronic, leads to an increase in overall numbers of myofibroblast-like activated HSCs that are actively producing matrix, while simultaneously preventing degradation of the matrix through expression of TIMPs. It has been consistently accepted that increased production and activity of TIMPs are required for decrease in MMP activity (Perez-Tamayo et al., 1987, Benyon and Iredale, 2000). The key events in the reversion of liver fibrosis include apoptosis of HSCs, increased MMP activity, decreased expression of TIMPs, and increased degradation of collagen (Woessner, 1991). In the liver, myofibroblasts derived from hepatic stellate cells undergo apoptosis during the spontaneous resolution of liver fibrosis induced by CCl₄ treatment (Iredale et al., 1998). During spontaneous recovery from liver fibrosis, TIMP-1 level decreases, and collagenase activity and the apoptosis of hepatic stellate cells increase, which results in an increased fibrolysis and removal of excessively deposited ECM. MMPs could be derived either from activated HSCs prior to or during apoptosis, from quiescent HSCs, or from other liver cells (Iredale, 2001). It is still unknown how the fibrotic tissues are removed in vivo and how MMPs, which are usually down-regulated in established fibrotic tissues, are expressed again during the resolution process (Han, 2006). Our result showed that CCl₄-intoxication decreased MMP-9 expression in CCl₄ group 72 h after toxin discontinuation, with further decrease in the CCl₄ control group 2 weeks later. Luteolin therapy has induced the expression of MMP-9, with the highest increase in the group treated with 50 mg/kg. The MMP-9 production by other cells distinct from HSCs could explain its increased expression despite HSC deactivation. In contrast, MMP-2 did not play a significant role in this model, which was confirmed by immunohistochemistry (data not shown).

The mechanisms by which MT participates in the pathology and the reversion of liver fibrosis are still largely unknown. Although a number of biological functions have been proposed for MTs, most of them are related to its metal-binding property, mainly Zn and Cu homeostasis and heavy metal detoxification (Andrews, 2000). Recently, we have shown dose- and time-dependent up-regulation of MTs expression by luteolin in acute hepatotoxicity induced by CCl₄ and the enhancement of hepatic regenerative capability (Domitrić et al., 2008a). Hepatic MT I/II expression in necrotic areas was correlated with the Zn and Cu content. However, MTs expression in chronic exposure to CCl₄ in this study was not correlated with either the Zn or Cu hepatic content. Increased MT synthesis also occurs in response to oxidative stress (Chubatsu and Meneghini, 1993). In this study, disturbance in antioxidative status in the CCl₄ control group and groups treated with luteolin was not observed, therefore we could not explain the down-regulation of MT I/II as a consequence of oxidative stress. Furthermore, MT has the essential role in cell proliferation and liver regeneration (Apostolova and Cherian, 2000). Immunohistochemical studies have demonstrated increased MT expression both in the cytoplasm and nucleus of rapidly proliferating cells (Miles et al., 2000). MT may donate Zn and Cu to certain metalloenzymes and transcription factors associated with gene transcription, cell replication, differentiation, and growth (Włostowski, 1993, Coyle et al., 2002). Jiang and Kang (2004) have suggested that gene therapy with MT could improve the recovery from liver fibrosis. The same authors have shown that mice which have developed a reversible liver fibrosis upon removal of CCl₄ had a high level of hepatic MT, but mice which developed an irreversible fibrosis, had low expression of MT. Therefore, hepatic MT expression could reflect the severity of chronic liver damage, showing down-regulation in injured tissue (Carrera et al., 2003). The results of this study show that the MT I/II expression was progressively down-regulated from the CCl₄ group to the CCl₄ control group. The level of MTs in livers was related to the progression of CCl₄-induced fibrosis and a complete down-regulation of MT I/II in the CCl₄ control group indicates development of an irreversible fibrosis. On the other hand, the positive correlation of MT I/II expression with luteolin dosage and liver tissue repairment suggests its direct involvement in the regeneration of liver tissue. The liver sections from mice treated with luteolin 50 mg/kg have shown strong up-regulation of MT I/II, including nuclear expression, which was accompanied with a complete resolution of fibrotic scar tissue, suggesting the pronounced therapeutic effect of luteolin. Our results support the view that MT I/II could be the factors associated with a favorable clinical outcome in the response to therapy (Carrera et al., 2003).

Regardless of these interesting and important findings, there are some limitations of the present study. The mechanistic insights into the luteolin-induced recovery of irreversible liver fibrosis need to be further approached. Although the important parameters of fibrosis and liver regeneration were determined, other factors that are possibly involved in the liver fibrinolysis need to be explored.

In conclusion, the luteolin treatment has induced a complete reversion of established liver fibrosis by decreasing hepatic fibrogenic potential, and stimulated MT I/II expression in the liver in a dose-dependent manner, suggesting the strong enhancement of hepatic regenerative capability. Importantly, the results obtained from this study suggest the therapeutic application of luteolin in patients with hepatic fibrosis, although further studies, in a more chronic model of liver fibrosis, are required.

Conflict of interest statement
The authors declare no conflict of interest.

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